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Small RNAs: Emerging key players in DNA double-strand break repair

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DNA double-strand break (DSB) is the most deleterious form of DNA damage and poses great threat to genome stability. Eukaryotes have evolved complex mechanisms to repair DSBs through coordinated actions of protein sensors, transducers, and effectors. DSB-induced small RNAs (diRNAs) or Dicer/Drosha-dependent RNAs (DDRNAs) have been recently discovered in plants and vertebrates, adding an unsuspected RNA component into the DSB repair pathway. DiRNAs/DDRNAs control DNA damage response (DDR) activation by affecting DDR foci formation and cell cycle checkpoint enforcement and are required for efficient DSB repair. Here, we summarize the findings of diRNAs/DDRNAs and discuss the possible mechanisms through which they act to facilitate DSB repair.

DNA damage, small RNAs, diRNA, Dicer, Argonaute

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Eukaryotic genomes are continuously challenged by a variety of extrinsic or intrinsic insults including environmental genotoxic agents or those generated spontaneously during cellular metabolism. These factors can induce various DNA lesions including DNA double-strand break (DSB). DSB is the most deleterious form of DNA damage and can cause mutation, genome instability, and cell death, if not properly repaired [1,2]. To achieve efficient and faithful repair of DSB, cells have evolved complicated DNA damage response (DDR) and multiple DSB repair pathways [1,2].

DDR is a signal transduction pathway that detects, amplifies, and transduces DNA damage signal to the whole cell to elicit cellular responses [1]. Detection of DSBs triggers the activation of damage sensor proteins including phosphatidylinositol 3-kinase-like protein kinases (PIKKs), ATM, ATR, and DNA-PK. These proteins initiate a signaling cascade via phosphorylating many downstream targets [2]. Histone variant H2AX is one of the key substrates of these sensor kinases. Phosphorylated form of H2AX (γ-H2AX) accumulates around DSB sites and may provide a scaffold for the recruitment of DDR proteins and chromatin remodeling complexes to amplify damage signal and promote DSB repair [3−5]. DSBs are repaired by two major pathways: nonhomologous end joining (NHEJ) and homologous recombination (HR). These two repair pathways all require well-regulated enzymatic actions of protein sensors, transducers, and effectors [2,6−8].

Over the last decade, small RNAs of ~20–30 nucleotide (nt) have emerged as key players in various biological processes. In eukaryotes, three major classes of small RNAs have been discovered: microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs) [9,10]. miRNAs and siRNAs are widespread in animals and plants. They are processed from double-stranded RNA

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(dsRNA) precursors by RNase III domain-containing Dicer or Dicer-like (DCL) proteins. They associate with members of the Argonaute (AGO) family and mediate gene expression at the transcriptional or post-transcriptional level [9,10]. piRNAs are animal-specific small RNAs that are generated from single-stranded RNA (ssRNA) precursors in a Dicer-independent manner [11–13]. They primarily function in the germ line to maintain genome stability by repressing transposons and repetitive elements [10,14,15]. Functionally analogous to animal piRNAs, plant heterochromatic siRNAs (hc-siRNAs) are produced from transposons and repetitive sequences and associated with AGO4 subfamily members [16]. They can direct DNA methylation and hence trigger transcriptional gene silencing through a pathway known as RNA-directed DNA methylation (RdDM) [17,18].

Recently, we and others have discovered a new class of small RNAs that is involved in DSB repair in plants and animals. In this minireview, we summarize the discovery and functions of these DSB repair-related small RNAs, and discuss the possible mechanisms through which these small RNAs function in the DSB repair pathway.

1 The discovery of DSB-induced small RNAs

In an attempt to explore whether small RNAs could play a role in DSB repair, we found that DCL proteins, which are responsible for small RNA biogenesis in *Arabidopsis*, are required for efficient DSB repair, suggesting the involvement of small RNAs in this process [19]. Indeed, Northern blot and deep sequencing analyses detected the production of small RNAs from the sequences around the DSB site. These small RNAs were named diRNAs for DSB-induced small RNAs. Further genetic analyses demonstrated that the biogenesis of diRNAs requires not only the classical small RNA pathway components, including DCLs, DNA-dependent RNA polymerase IV (Pol IV), and RNA-dependent RNA polymerases (RDR2 and RDR6) but also a PI3 kinase ATR that is known to be involved in DDR [19]. This reveals a genetic link between DDR and small RNA biogenesis.

In light of the conservation of both small RNA and DSB repair machineries between plants and animals, we extended diRNA analysis to human cells. We found that, as in *Arabidopsis*, DSBs in human cells induced diRNA production from the sequences close to the DSB sites [19]. Soon after the finding of diRNAs, d'Adda di Fagagna and colleagues identified a class of DICER- and DROSHA-dependent small RNAs (named DDRNAs) that is involved in DDR [20]. They found that DDR is greatly impaired in DICER- or DROSHA-deficient cells, suggesting that some DICER and DROSHA products are required for DDR activation. They further found that DICER- and DROSHA-dependent small RNAs (DDRNAs) are generated from an engineered damage site. More importantly, they demonstrated that introduction of *in vitro* synthesized sRNAs that matching the damage site can rescue DDR foci formation, indicating a role for DDRNAs in DDR [20]. As miRNAs are also DICER- and DROSHA-dependent, we suggest using the term diRNA instead of DDRNA for these small RNAs that are involved in DNA damage response and repair. In another work by Michalik et al. [21], the authors described the generation of one small RNA response at DNA ends in *Drosophila* cells. They deep sequenced small RNAs from cells transfected with linearized and circular plasmids and found that compared with the circular plasmid, linearized plasmids generated about 10 times more small RNAs from the break site at both strands. This response is specific to a DSB since nicked DNA structures do not trigger endo-siRNAs production.

2 When, where, and how diRNAs function in DSB repair?

The identification of diRNAs in both plants and animals reveal a conserved role for small RNAs in DSB repair. This adds an unsuspected RNA component to the DSB signaling and repair pathway, which was previously considered to be made up exclusively of proteins. The molecular mechanisms through which diRNAs function in DSB repair remain largely unknown.

The accumulation of phosphorylated H2AX (γ-H2AX) formation around DSB sites is one of the earliest events in response to DSB and facilitates local recruitment and retention of additional DDR factors [2,3,5]. Depletion of diRNAs has no impact on γ-H2AX focus formation [19,20], indicating that diRNAs are not involved in γ-H2AX accumulation. d'Adda di Fagagna and colleagues [22–27] showed that diRNAs are required for MDC1 and 53BP1 foci formation, processes downstream of γ-H2AX. These observations suggest that diRNAs function downstream of or in parallel to γ-H2AX to recruit DDR components onto DSB site to facilitate its repair.

diRNAs are specifically produced from the regions close to the DSB sites. This raises a possibility that diRNAs provide the specificity of DSB repair, and such specificity may be achieved by base-pairing between diRNAs and the damaged DNA or scaffold transcripts made from the damage sites. The findings of damage-induced aberrant RNAs in *Neurospora crassa* [28] as well as the requirement of Pol V for DSB repair in *Arabidopsis* [19] suggest that the pairing very likely occurs between diRNAs and scaffold transcripts (Figure 1). It will be of great interest to detect the proposed scaffold transcripts and to determine whether the production of such transcripts is induced by DSB.

Small RNAs invariably associate with AGO family proteins to execute their function. In *Arabidopsis*, diRNAs interact with a DNA damage-inducible AGO2 [19]. A muta-

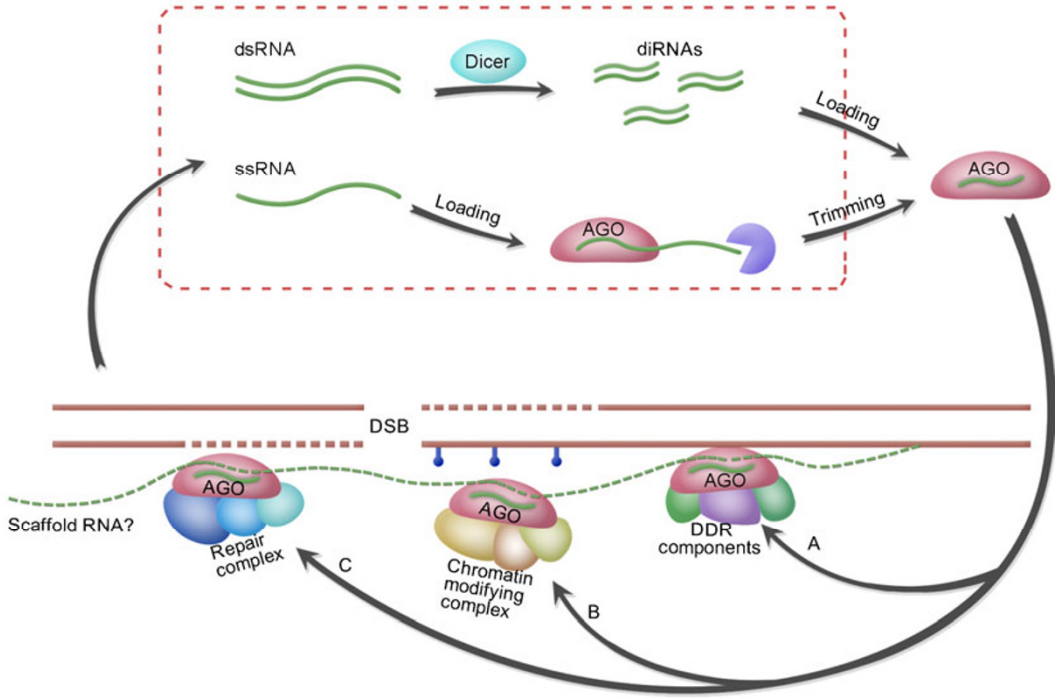

Figure 1 A model for diRNA-mediated DSB repair. At a DSB, single-stranded RNA transcripts (ssRNAs) are generated by RNA polymerases (RNA polymerase IV in *Arabidopsis*). ssRNAs are then converted into double-stranded RNAs (dsRNAs) via the activity of RNA-dependent RNA polymerase (RDR) or bidirectional transcription. dsRNAs are processed by Dicer into diRNAs, which are subsequently incorporated into Argonaute (AGO) protein (AGO2 in *Arabidopsis*). Alternatively, long ssRNAs can be directly loaded into AGO protein and processed by 3′ to 5′ exonucleolytic trimming. In either case, AGO/diRNA complexes are localized to the DSB site through interaction with putative scaffold RNAs that are produced from the sequences around the DSB site. There, AGO/diRNA complexes may recruit DNA damage response (DDR) components to activate DDR (A), chromatin modifying complex to modify local chromatin (B), or directly recruit DSB repair complex to the DSB site (C) to facilitate DSB repair.

tion in AGO2 dramatically reduces DSB repair efficiency [19]. Similarly, depletion of Ago2 in human cells significantly decreases DNA repair [19]. This indicates that diRNAs function in the form of complexes with AGO proteins. We hypothesize that diRNA- or DDRNA-programmed AGO complexes may interact with and recruit DSB signaling and/or repair components to DSB sites to mediate DDR and/or DSB repair. This hypothesis is supported by the finding that accumulation of MDC1, 53BP1 and other DDR signaling components at DSB sites is dependent on diRNAs [20]. There are many more downstream effector proteins that are recruited to DSB sites to facilitate repair. For example, RAD51 accumulates and forms presynaptic filament on the resected ssDNA with the assistance of mediators such as BRCA2 [7,29,30]. With the discovery of diRNAs, it is tempting to speculate that the effector proteins are guided by diRNAs to search and accumulate onto the damage sites (Figure 1). It will be very interesting to investigate whether diRNA-bound AGO proteins interact with any proteins in the DSB repair pathway and whether the recruitment of these repair proteins to DSB sites is dependent on diRNAs.

Alternatively, in the light of the accumulating evidences that DSBs trigger a number of chromatin microenvironment changes including DNA methylation, histone modifications, and chromatin remodeling around the DSB sites [27,31,32], it is officially possible that diRNAs may direct these chromatin changes at the DSB sites, through a similar mechanism as RdDM [17]. In *Arabidopsis*, diRNAs do not seem to function through RdDM to mediate DSB repair [19]. However, we cannot exclude the possibility that diRNAs-mediated DSB repair involves DNA methylation changes through other mechanisms. It is known that histone variants of damaged chromatin are modified by methylation [33–37], acetylation [38–41], and ubiquitylation [42–47]. These histone modifications play important roles in the accumulation and function of DDR proteins including MDC1, 53BP1, and BRCA1 [35,42–45]. DiRNAs play a role in MDC1 and 53BP1 foci formation at DSB sites [20]. It would be interesting to examine whether such role for diRNAs is achieved through mediating chromatin modifications.

The discovery of diRNAs reveals an unsuspected novel function for small RNAs and adds another layer of regulation in the DNA damage repair pathway. We anticipate that the finding of diRNAs will trigger enormous interests in researching the mechanism of diRNA-mediated DSB repair.

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